LAB NOTEBOOK YEAST TEAM

Day 07/07

Subculuring of just received yeast

Preparation of YPD medium :
27,5mL of 2M glucose solution in 500mL of YPD medium already made to have 2% solution.

Subculturing in 4 erlenmeyer, each of them contain 25mL of sowed medium.
Growth at 30°C, 4700 rpm.

Transformation of E.coli with pYES2

Day 08/07

Subculturing of E .coli with pYES2 in 4 bacto tube with 5 mL LB medium. 22ML of LB medium were prepared from the stock solution by adding 22µL of Amphicilin 50 mg/mL

Day 09/07

miniprep E.coli with pYES2
using qiaprep spin miniprep kit

nanodrop essay
4 tubes PYES2 en ng/uL in 50uL
1 10,0 ng/uL
2 6,7 ng/uL
3 5,7 ng/uL
4 11,9 ng/uL

Subculturing E.coli with PYES2

Subculturing of E .coli with pYES2 in 4 bacto tube with 5 mL LB medium. 22ML of LB medium were prepared from the stock solution by adding 22µL of Amphicilin 50 mg/mL

Day 10/07

DNA extraction

OD yeast (1st tube)
3,81 Absorbance 360nm
1,21 à 600nm

lysis solution
add 5uL of SDS à 20% in 100µl H2O

0,13g in 10mL H2O de LiAc
500µL in 10mL H2Ode SDS

Procedure

1. 100–200 µL liquid yeast culture (OD_{600}=0.4). Suspend cells in 100 µL 200 mM LiOAc, 1% SDS solution.

2. Incubate for 5 min at 70°C. 11h01 16s à 11h06 16s

3. Add 300 µL 96–100% ethanol, then vortex.
4. Spin down DNA and cell debris at 15,000× *g* for 3 min.

5. Wash pellet with 70% ethanol.

6. Dissolve pellet in 100 µL H_2O or TE and spin down cell debris for 15 s at 15,000× *g*.

7. Use 1 µL supernatant for PCR.

Surpenantent was transfer (200uL) in 4 tubes ***Y1-4***

Tube	ADN ng/uL	Ratio 260/280	ratio260/230
Y1	146,7	1,76	0,70
Y2	160,4	1,78	0,73
Y3	157,8	1,77	0,70
Y4	162,6	1,77	0,71

second DNA extraction on surpernatent 100µl (same procedure)

Y5	72,6	1,86	0,88

Y6	90,5	1,78	0,73

miniprep PYES2
qiaprep spin miniprep kit

PYES2 50 µl in each tube

6,7 ng/µL
8,8 ng/µL
10,4 ng/µL
5,4 ng/µL

Day 13/07

curdlan extraction

on 100 µl de levure
add 100 µL 200 mM LiOAc, 1% SDS
70° during 5min

add 100µl NaOH 0,5M during 4h
centrifugation
discard supernanent

1. 100uL liquid yeast culture Suspend cells in 100 µL 200 mM LiOAc, 1% SDS solution.
2. Incubate for 5 min at 70°C.
3. Add 300 µL 96–100% ethanol, then vortex.

Supernanent is collected for later analysis.

Day 14/07

Curdlan Assay.

solution aniline blue 0,1%

0,01g in 10mL water

Preparation DYE MIX :

40 volumes of 0.1% aniline blue in water

21 volumes of 1 N HCl

59 volumes of 1 M glycine/NaOH buffer pH 9.5

For a final volume 3530ul of DYE MIX

1200 µL aniline blue 0,1%

630 µL HCl

1770 µL buffer 1M glycine NaOH 1M pH 9,5

Final Volum 3530ul of DYE MIC

Procedure

1) Add 200 µl 1 N NaOH to 100µL of sample for a final volume of 300 µL in a 1.5 mL microcentrifuge tube.

30µL of dilued sample in 270µL of NaOH 1N

2) Add 30 µL of 6 N NaOH

3) Incubated at 80 °C for 30 min.

4) The tube was immediately put on an ice bath.

5) Add 630 µL DYE MIX* into the tubeand mix

6) Incubated at 50 °C for 30 min

7) The unbound fluorescent dye was decolorized at room temperature for 30 min

8) Mesure the fluorescence intensity with a fluorescence spectrophotometer

- emission wavelength of 502 nm (20 nm slit width)

- excitation wavelength of 398 nm (20 nm slit width).

Notes:

The dilution factor for tested samples was determined empirically to obtain fluorescence intensity

within
the calibration range.
Each sample was performed in duplicate.
Curdlan was used as 1,3--G standard to construct the calibration curve.

Day 15/07

DYE mix

630 uL HCl
1770 uL buffer 1M glycine NaOH 1M pH 9,5
1200 uL aniline blue 0,1% 0,01g dans 1mL

Final volume 3600µL

subculturing yeast
2x 25mL YPD+glc in 2 50mL erlenemeyer
OD monitoring every 6h (except during the night)

Day 16/07

Curdlan assay

calibration range construction

0,3mg of curdlan is dissolved in 10mL NaOH 1N : solution T1
By succesive dilution we make all the calibration range

	V en uL T-1	V NaOH à 1M	Concentration µg/mL
T1			30
T2	666	334	20
T3	750	250	15
T4	666	334	10
T5	500	500	5

Bacteria +pYES2 and Yeast cultur stock

850µL bacteria pYES2 + 150µL glycérol put at -80°C

Curdlan extraction

sonication lysis of yeast cells

3x 10mL of cultur (OD 0,159) was remove in a falcon tube

Sonication lysis each tube were sonicated during 10s, 20s 30s respectively (stop during 30s in ice every half time.)

2x 100uL were taken from each tube

centrifugation 3min 15000g to spin down DNA
surpernatent is collected.
Add100uL NaOH 0,5M to supernanent, leave it at room temperature overnight

Competent yeast Preparation.

Measur OD of yeast cultur :, 2,49 pour le repiquage de Y2.

1) Inoculate the yeast strain to be transformed in 10 ml of YPD medium and
incubated at 30 ° C with stirring overnight.
2) Dilute 1:50 the overnight culture into 50 ml of YPD medium.
3) Grow at 30 ° C under agitation until a cell density of 1 to 3 x 107
cells / ml. It takes 10 ml
a culture 107cells / ml for transformation.
4) Count the cells under the microscope with hemocytometer .
- Dilute the culture 1:10 in an Eppendorf tube (50 .mu.l of cells + 450 .mu.l
of distilled water)
- Transfer about 30 .mu.l of this dilution between slide and cover slip
- Count the cells 20 small squares in the microscope and to average
- Calculate the cell concentration knowing number of cells of a square x = concentration of 105 cells / ml (do not forget the dilution factor)
5) Centrifuge culture at 4 ° C for 5 min at 5000 rev / min in Falcon tubes.
/ 1215
6) Perform 1 wash gently resuspending the pellet with a pipette in 20 ml of TE solution / LiAc sterile.

7) Centrifuge at 4 ° C for 5 min at 5000 rev / min.
8) Resuspend pellet in order to have 2 x 109
cells / ml in the solution TE / LiAc
(50 .mu.l per 10 ml of culture at 10
7
cells / ml) (consider the volume of the pellet).
9) Incubate at 30 ° C for 15 minutes without stirring.
10) Freeze part of yeasts competent

Day 17/07

Curdlan assay

10 sample: 6 sonication lysis, 4 chemical lysis (LiAc)

two calibration range

5 concentrations : 30; 20; 15; 10; 5µg/mL for the first calibration range T 1-5

5 concentration 0,3 ; 0,2 ; 0,15 ; 0,10 ; 0,5 for the dilued calibration range dilué T' 1-5

1) 30uL sample dilued with 270µL NaOH 1N

2) Add 30 µL of 6 N NaOH

3) Incubated at 80 °C for 30 min.

4) The tube was immediately put on an ice bath.

5) Add 630 µL DYE MIX* into the tubeand mix

6) Incubated at 50 °C for 30 min

7) The unbound fluorescent dye was decolorized at room temperature for 30 min

8) Mesure the fluorescence intensity with a fluorescence spectrophotometer

- emission wavelength of 502 nm (20 nm slit width) -
- excitation wavelength of 398 nm (20 nm slit width).

Calibration range.

	V en uL T-1	V NaOH à 1M	[curdlan] ug/mL
T1			30
T2	666	334	20
T3	750	250	15
T4	666	334	10
T5	500	500	5

	V en uL T-1	V NaOH à 1M	[C] ug/mL
T'1			0,30
T'2	666	334	0,20
T'3	750	250	0,15
T'4	666	334	0,10
T'5	500	500	0,05

96 well-plate plan.

	1	2	3	4	5	6	7	8	9	10	11	12
A	/	/	/	/	T1	T2	T3	T4	T5	T1'	T2'	T3
B	/	/	/	/	/	T4'	T5'	10	10	20	20	30
C	30	Y	Y	Y	Y							
D												
E												
F												
G												
H												

Y Y extraction DNA

miniprep pYES2

Dosage au nanodrop :

	ng.uL-1	260/280	260/230
pYes 2 a	146,6	1,89	2,26
pYes 2 b	254,2	1,80	2,29
pYes 2 c	192,1	1,88	2,25
pYes 2 d	159,5	1,89	2,26

Day 20/07

DYE MIX preparation

480uL aniline blue 1%
252uL HCl
708 uL Glycine NaOH buffer

Chemical lysis procedure
take 10mL yeast cultur
add 10mL LiAc
heat at 70°C during 10min
add 10mL NaOH
overnight at 37°C

Day 21/07

curdlan extraction
same procedure as usual

Day 22/07

purification curdlan V1

on 4x500µL of cultur

1) The culture was mixed with an equal volume of 0,5 N NaOH and lysis buffer (LiAC 200mM) and then centrifuged at 10,000 rpm for 10min to remove the cells.

2) The resulting supernatant was neutralized by adding 3 N HCl

3) The precipitate formed was collected by centrifugation at 10,000rpm for 10min.

-It was washed three times with water by centrifugation, and then dehydrated with acetone and dried in vacuo.

4) This precipitated polymer is the curdlan-type polysaccharide.

A control was made at each step

Purification Curdlan V2

on 4x2mL of cultur

1. Centrifugation of the culture at 12000 g for 30 min
2. resuspension of the pellet in an equal volume of 0.5 N sodium hydroxide at 4 ° C
3. Stir for 10 min at 4 ° C
4. Allow to stand for 3 hours at 4 ° C
5. Centrifugation of the resulting solution at 12,000 g for 40 min
6. Precipitation of Curdlan (located in the supernatant) by neutralization with acetic acid (10%)
7. washing with water , acetone and ether

PCR with both primer EX-SP and Not1 Bmh1

Day 23/07

purification du curdlan V1

on 4x100µL of crultur

same procedure

PCR with Not1 BmH1 primer

agarose gel for verfication

Day 24/07

pas de précipitation observé après purification du curdlan.

PCR both primer + electrophorèse

extraction génomic DNA yeast McClean: Genomic DNA Prep

Transfer 2x500µL of liquid culture of yeast grown for 20 – 24 h at 30°C in YPD into a microcentrifuge tube. Pellet cells by centrifugation at 20,000 × g for 5 minutes.

1. Add 500 µl of lysis buffer (, 2% SDS, 200mM LiAc, 1 mM EDTA, pH 8.0).
2. Immerse tubes in a dry ice-ethanol bath for 2 minutes, transfer to in a 95°C water bath for 1 minute. Repeat; vortex 30 seconds.
3. Add 66 µl of chloroform; vortex 2 minutes.
4. Centrifuge 3 minutes, room temperature, 20,000 × g.
5. Transfer the upper aqueous phase to a microcentrifuge tube containing 400 µl ice-cold 100% ethanol. Mix by inversion or gentle vortexing.
6. Incubate at room temperature, 5 minutes. Alternatively, precipitate at -20°C to increase yield.
7. Centrifuge 5 minutes, room temperature, 20,000 × g. Remove supernatant with a pulled Pasteur pipette by vacuum aspiration.
8. Wash the pellet with 0.5 ml 70% ethanol, spin down as described in step 8 above. Remove supernatant.
9. Air-dry the pellets at room temperature or for 5 minutes at 60°C in a vacuum dryer.
10. Resuspend in 25–50 µl TE [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0)] or water. Samples obtained directly from plates should be resuspended in a 10 µl volume, because the yield will be smaller. 0.25 µl RNase cocktail (Ambion) should be added to the samples used for Southern blot hybridization (final concentration 0.125 U RNAse A, 5 U RNase T1).

subculturing yeast (100µL of liquid medium in 10 mL of YPD fresh medium)

Day 27/07

extraction DNA yeast

on 1,5 mL cultur

Add 1,5 mL Lysis buffer 200mM LiAc 1%SDS, 1mM EDTA

Add 200 µl of chloroform; vortex 2 minutes.
Centrifuge 3 minutes, room temperature, 20,000 × g.
Transfer the upper aqueous phase to a microcentrifuge tube containing 400 µl ice-cold 100% ethanol. Mix by inversion or gentle vortexing.

Incubate at room temperature, 5 minutes. Alternatively, precipitate at -20°C to increase yield.
Centrifuge 5 minutes, room temperature, 20,000 × g. Remove supernatant with a pulled Pasteur pipette by vacuum aspiration.
Wash the pellet with 0.5 ml 70% ethanol, spin down as described in step 8 above. Remove supernatant.
Air-dry the pellets at room temperature or for 5 minutes at 60°C in a vacuum dryer.
Resuspend in 25uL water 1mM EDTA 2,5ul (pH 8.0)] or water. Samples

4 tubes

Y1' et Y2' (first extraction)

et 2*Y2 ADN (second extraction)

dosage nanodrop

		260/280	230/280
Y2 ADN	0,8 ng/uL	-1,41	0,59
Y2 ADN	9,9	1,99	0,65
Y1'	26,9	1,83	0,7 0,70
Y2'	23,7	1,88	0,67 0,67

Curdlan Assay

16 tubes

6 tubes from Cudlan purification V1; 3 from cultur (unpurified) and 3 others form lysis extract à

4 tubes de T1-2 et T1-2' Control at each step

4 tubes from curdlan purification V2 from cultur

2 tubes from supernatent and 1 tube from pellet resuslting from the curdlan purificatio V2 procedure

As usual for the calibration range

PCR with both primers

Day 28/07

extraction DNA yeast
4x1,5mLof cultur

DNA assay from nanodrop analysis

µg/mL	260/280	230/280
21,9	1,73	0,75
26,4	1,96	0,89
16,6	1,83	0,80
33,6	1,96	0,89

Day 29/07

PCR with EX-SP primer on new DNA extraction

Sequential digestion with ApaI et BgLII de l'ADN génomique de levure

We want to cut genomic DNA to improve PCR efficiency

20µl DNA (1µg)
40µl buffer cut smart
1 µl ApaI
320 µl dd H2O
4h at 37°C
400µl buffer 3,1
1µl BglII

Day 31/07

Transformation in bacteria and amplification of pFA6a-His3MX6-PGAL1-3HA

Day 03/08

Sequential digestion

Preparation cultur medium 2xYPD 500mL and 2xLB 500mL

miniprep x6 pFA6a-His3MX6-PGAL1-3HA

DNA assay

1 - 31,2 ng/uL
2 - 35,5
3 - 24,1
4 - 42
5 - 26,9
6 - 51

Day 04/08

Sequential Digestion

YPD plate were made

Day 05/08

Launch 2 YPD liquid culture 25ml + AMP (25µl)
sowing Petri plate YPD + AMP with colony on cone in 30µl water (10ul in each)

PCR by PCR of Derek's Cassette (His-Gal)

Day 07/08

Yeast Transformation

HIGH EFFICIENCY YEAST TRANSFORMATION

For approximately 5-10 transformations:
Grow 100 mls of cells overnight to an optical density of between 0.5 and 1.

Prepare LiOAc MIX and PEG MIX ahead of time.

1. Split culture into two 50 ml conical tubes, pellet the cells, and remove the supernatant.

2. Resuspend pellets in a total of 15 mls LiOAc MIX . Combine into a 15 ml tube, pellet the cells, and remove the supernatant.

3. Resuspend pellets in 15 mls LiOAc MIX , pellet the cells, and remove the supernatant.

4. Resuspend the pellet in 0.5 - 1 mls LiOAc MIX.

5. Set up transformations. To each transformation add:

   30 ul 5 mg/ml Salmon Sperm DNA
   1-5 ug specific DNA (1 ul of miniprep DNA)
   vortex
   add 100 ul cell suspension
   vortex
   add 0.7 ml PEG MIX
   vortex

6. Incubate 60 min., 30 degrees C.

7. Heat shock 20 min., 42 degrees C.

8. Pellet cells and resuspend in 150 ul YPD.

9. Plate on selective media. Milieu minimum hist- gal+

LiOAC MIX:
100 mM LiOAc
10 mM Tris (7.5)
1 mM EDTA

PEG MIX:
40% PEG, mw 3350
100 mM LiOAC
10 mM Tris (7.5)
1 mM EDTA

Day 10/08

transformant contamination with fungus ?
Making some plates YNB – His + Gal

Transformation reisolationon YNB –His + Gal

Day 11/08

Curdlan prufication

Day 13/08

DNA Extraction with tranformants

Day 17/08

DNA nanodropping

	260/280	260/230	
R 444,9 ng/ul	1,91	2,01	
W712,8 ng/ul	1,45	1,36	RNA and organic molecules contamination

PCR check
Derek primers check

2 YPD gal pseudo tranformants red and white 200ml
200 ml YPD with wT

Transformation

Day 18/08

Purification of the Derek plasmid
DNA Extraction on colonies and liquid cultures
W R Wl Rl

Day 19/08

PCR check on red and white colonies
Testing on agarose 1% gel

Liquid cultures with YPD +GAL +Amp
erlenemeyer 20ml red and white colonies

Petri box YPD amp with wT

PCR extraction FKS1 not1 bamh1 on Dereck DNA Extraction
and PCR check with GAL sens and FKS1 antisens on colony.

Day 20 /08

Testing PCR on agarose 1% gel.

SC= ADN derek + amorces not1 bmh1

Day 22/08

-manufacturing solid medium LB + chloramphenicol at 10ug / mL
-PCR FKS1 not1 BamH1 extraction from the purified DNA extraction control
-and check with GAL PCR sense and antisense FKS1 on solid and liquid red colony.

PCR check with new transformants
new transformation negactif witness +

Launch of inoculum liquid culture in a volume of 6 mL (x 6) + Gal medium YPD

Day 24/08

- Launching cultured in 250 ml Erlenmeyer flask, to partier 1 mL inoculum of OD 0.2. There are two Wild type and Transforming conditions. This experiment is carried out in triplicate with a time interval of 15 min.
-New DNA purification, on new transformants
-PCR Verification processing (check) OK

Day 25/08

-Levy culture medium at different time to see a growth rate difference. It should be noted that the transformant grows faster and thus will more rapidly in the stationary growth phase.
-PCR on plasmid-pFA6a His3MX6-PGAL1-3HA with the primers for assembly Gibson method. Good result.

Day 26/08

-Dosage On crops without lysis and purification. the witness is the witness made containing culture medium alone because it contains YEAST EXTRACT that can give a false positive.
-The Result is that the witness is equal to the sample so glucans are only visible after lysis and purification.
-Gibson -Method assembly of the HIS fragment - GAL and linearity plasmid pSB1C3

Day 27/08

-Lysis of all the remaining early purification cultures.
-Transformation with plasmid pSB1C3 - His-Gal

Day 28/08

The transformation market.
setting inoculum culture with the transformants for a mini-prep
Purification end of the previous day and Determination of glucans after cell lysis and after final purification. The result is that we lose 90% of glucan in purifying and transforming our produce less than wild-type glucan, so the gene was affected and off.